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(57) Abstract

The present invention relates generally to the immunodetection of oxidatively-modified low density lipoproteins (LDL), antibodies thereto or an immune complex thereof, in biological fluid. More particularly, the present invention relates to an enzyme-linked immunosorbent assay (ELISA) technique for the analysis of oxidatively-modified LDL, antibodies thereto or an immune complex thereof in mammalian plasma, and even more particularly, human plasma. The present invention is also directed to a kit to facilitate the ELISA technique. The present invention is particularly useful in the assessment of coronary heart disease risk.

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IMMUNOLOGICAL ASSAY OF OXIDATIVELY MODIFIED HUMAN LOW DENSITY LIPOPROTEINS IN PLASMA

The present invention relates generally to the immunodetection of oxidatively-modified low density lipoproteins (LDL), antibodies thereto or an immune complex thereof, in biological fluid. More particularly, the present invention relates to an enzyme-linked immunosorbent assay (ELISA) technique for the analysis of oxidatively-modified LDL, antibodies thereto or an immune complex thereof in mammalian plasma, and even more particularly, human plasma. The present invention is also directed to a kit to facilitate the ELISA technique. The present invention is particularly useful in the assessment of coronary heart disease risk.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

Plasma low density lipoprotein (LDL) can undergo free radical induced oxidation either catalyzed by divalent ions such as Cu^{2+} (Steinbrecher et al. I Proc. Natl. Acad. Sci. U.S.A. 89:3883-7, 1984) or by incubation with cultured cells such as endothelial cells (Steinbrecher et al. supra; Moreal et al. Arteriosclerosis 4:357-364, 1984), vascular smooth muscle, (Morel et al. supra) monocytes, neutrophils (Cathcart et al. J.Leukocyte Biol 38:341-350, 1985) or macrophages (Parthasarathy et al. Arteriosclerosis 6:505-510, 1986). The oxidation of LDL results in a number of chemical changes including increased electrophoretic mobility (Steinbrecher et al. supra), changes in fluorescent properties (Koller et al. FEBS Lett. 198:229-234, 1986), loss of polyunsaturated fatty acids and vitamin E and the generation of aldehydes (Esterbauer et al. J.Lipid Res. 28:495-509, 1987) and the modification of lysine residues of apolipoprotein B by lipid peroxide decomposition products (Steinbrecher et al. II J.Biol. Chem. 262:3603-3608, 1987). The functional changes of oxidized LDL include recognition by the scavenger receptor on macrophages

(Steinbrecher et al. II supra; Parthasarathy et al. Proc. Natl. Acad. Sci. U.S.A. 84:507-540, 1987), cytotoxicity to human vascular smooth muscle cells and endothelial cells (Hessler et al. Atherosclerosis 32:213-229, 1979), chemotaxis for human monocytes (Quinn et al. I Proc. Natl. Acad. Sci. U.S.A. 84:2995-2998, 1987) and inhibition of mouse peritoneal macrophage chemotaxis (Quinn et al. II Proc. Natl. Acad. Sci. U.S.A. 82:5949-5953, 1985).

It is now becoming clear that the oxidative modification of LDL by endothelial cells, smooth muscle cells, monocytes and macrophages in the arterial wall plays an important, if not the decisive, role in the initiation of the atherosclerotic lesion (Steinberg et al. Am. J.Cardiol. 62:6B-12B, 1988). Oxidized LDL attracts monocytes into the subendothelial space where the monocytes are converted to macrophages which then take up the oxidized LDL generating the foam cells characteristic of the early fatty streak. The mobility of the macrophages is inhibited, trapping them in lesion sites. The oxidized LDL is also toxic to endothelial cells and could be responsible for endothelial injury.

An oxidatively modified LDL subfraction has been isolated from human plasma in amounts ranging from 5% to 10% of the total LDL (Arogaro et al. Arteriosclerosis 8:79-87, 1983). Increased blood concentrations of cholesterol are a well-established risk factor for coronary heart disease (Castelli et al. Circulation 67:730-734, 1983). Oxidatively modified LDL also exists in an immune complex with antibodies. Free antibodies to oxidatively modified LDL also exist.

If oxidatively modified LDL is important in the pathogenesis of atherosclerosis then the measurement of oxidized LDL, antibodies thereto or an immune complex thereof in plasma may be a more reliable indicator of atherosclerotic risk than the measurement of cholesterol or LDL alone.

Accordingly, the present invention contemplates a method for detecting oxidatively-modified low density lipoprotein (LDL), or LDL in an immune complex with antibodies thereto, in biological fluid which method comprises contacting the

fluid to be tested with an antibody specific to oxidized LDL for a time and under conditions sufficient for an LDL-antibody complex to form and then detecting said complex by a detection means.

More particularly, the present invention is directed to a method for detecting oxidatively-modified LDL, or oxidized LDL in an immune complex with antibodies thereto, in human plasma which method comprises contacting the plasma to be tested with a monoclonal antibody specific to oxidised LDL, for a time and under conditions sufficient for an LDL-antibody complex to form and then detecting said complex by a detecting means.

Another aspect of the present invention relates to a method for detecting antibodies interactive with oxidized LDL or antibodies in an immune complex with oxidized LDL in biological fluid, such as human plasma, which method comprises contacting the fluid to be tested with oxidized LDL for a time and under conditions sufficient to form an antibody-LDL complex and then detecting said complex by a detection means.

In one embodiment, the detecting means comprises an antibody labelled with a reporter molecule capable of giving a detectable signal.

Yet another aspect of the present invention relates to a kit useful in the analysis of oxidatively-modified LDL, antibodies thereto or an immune complex thereof in human plasma.

The present invention also contemplates a method for assessing coronary heart disease risk in a human comprising quantitating the level of oxidatively-modified LDL, or oxidized LDL in an immune complex with antibodies thereto in plasma from said human by contacting said plasma with a monoclonal antibody specific to oxidized LDL for a time and under conditions sufficient to form an LDL-antibody complex and subjecting said complex to a quantifying detecting means and comparing the level so detected to a predetermined amount.

Another aspect of the present invention relates to a method for assessing coronary heart disease risk in a human comprising quantitating the level of antibodies specific for oxidatively-modified LDL or antibodies in an immune complex with oxidized LDL in plasma from said human by contacting said plasma with oxidized LDL for a time and under conditions sufficient to form an antibody-LDL complex and subjecting said complex to a quantifying detecting means and comparing the level so determined to a predetermined amount.

For convenience, "oxidatively-modified LDL" as used in the present specification has the same meaning as "oxidized LDL". Both terms are used interchangeably.

The present invention is directed to a method of detecting oxidatively-modified LDL, antibodies thereto or an immune complex thereof in biological fluid which comprises contacting the fluid to be tested with an antibody specific to oxidized LDL, or with oxidized LDL capable of binding to an antibody thereto for a time and under conditions sufficient for an LDL-antibody complex to form and then subjecting said complex to a detecting means. Henceforth, reference to oxidized LDL is intended to encompass oxidized LDL, antibodies thereto and/or an immune complex between said oxidized LDL and said antibodies. The latter complex is also referred to as a circulating immune complex. By "detecting" is meant to include both quantitative and qualitative detection of oxidized LDL or antibodies thereto. "Detecting means" includes, in the case of detecting oxidized LDL, a first antibody, specific or otherwise interactive to oxidized LDL and labelled with a reporter molecule capable of giving a detectable signal or a second antibody specific or otherwise interactive to said first antibody, where said second antibody is labelled with the reporter molecule. In case of detecting antibodies to oxidized LDL in a mammal (eg. human), the detecting means includes an anti-mammalian antibody capable of binding to the antibody to be detected, said anti-mammalian antibody labelled with a reporter molecule. "Biological fluid" is used in its broadest sense and includes in vitro culture supernatant fluid and/or cell extract or in vivo animal-, or more particularly, mammalian-derived fluid such as serum, plasma, tissue extract and the like. In one preferred embodiment, biological fluid is human plasma.

In accordance with the present invention, antibodies are generated to oxidatively-modified LDL. The latter refers to any LDL having undergone free radical induced oxidation either catalyzed by divalent ions such as Cu^{2+}, amongst others, or by incubation with cultured cells such as endothelial cells, vascular smooth muscle, monocytes, neutrophils or macrophages. Reference herein to oxidatively-modified LDL also includes reference to circulating populations of LDL subfractions such as from patients with cardiovascular disease. Oxidatively-modified LDL have properties as hereinbefore described. Although for the purposes of exemplification Cu^{2+} is described herein as being used to induce oxidation of LDL, this should not be construed as a limitation to a particular mode of oxidation. In the following disclosure, reference to oxidized LDL is also meant to encompass antibodies thereto and immune complexes thereof.

Both polyclonal and monoclonal antibodies are obtainable by immunization of animals with oxidatively-modified LDL, and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of the oxidatively-modified LDL, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in the present immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the produce. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example, Douillard and Hoffman, "Basic Facts About Hybridomas", in Compendium of Immunology, Bol. II, L. Schwartz (Ed.), 1981; Kohler and Milstein, I Nature 256:496-497, 1975; II European Journal of Immunology, 6:511-519, 1976).

Unlike preparation of polyclonal sera, the choice of animal is dependent on the availability of appropriate immortal lines capable of fusing with lymphocytes thereof. Mouse and rat have been the animals of choice in hybridoma technology and are preferably used. Humans can also be utilized as sources for sensitized lymphocytes if appropriate immortalized human (or nonhuman) cell lines are available. For the purpose of the present invention, the animal of choice may be injected with from about 20µg to about 500 µg of the oxidatively-modified LDL and preferrably 50 µg to about 200 µg. Usually the injecting material is emulsified in Freund's complete adjuvant. Boosting injections may also be required. The detection of antibody production can be carried out by testing the antisera with appropriately labelled antigen. Lymphocytes can be obtained by removing the spleen or lymph nodes of sensitized animals in a sterile fashion and carrying out fusion. Alternatively, lymphocytes can be stimulated or immunized in vitro, as described, for example, in Reading, J. Immunol. Meth. 53:261-291, 1982.

A number of cell lines suitable for fusion have been developed, and the choice of any particular line for hybridization protocols is directed by any one of a number of criteria such as speed, uniformity of growth characteristics, deficiency of its metabolism for a component of the growth medium and potential for good fusion frequency.

Intraspecies hybrids, particularly between like strains, work better than interspecies fusions. Several cell lines are available, including mutants selected for the loss of ability to secrete myeloma immunoglobulin. Included among these is the mouse myeloma line NSI (Kohler et al. III Eur. J. Immunol. 6:292-295, 1987). Another useful myeloma is P3-XAg8.653 (Kearney et at J. Immunol. 128: 1548, 1982) The present invention extends to subclones and other myeloma derived from P3-XAg8.653.

Cell fusion can be induced either by virus, such as Epstein-Barr or Sendai virus, or polyethylene glycol. Polyethylene glycol (PEG) is the most efficacious agent for the fusion of mammalian somatic cells. PEG itself may be toxic for cells, and various

concentrations should be tested for effects on viability before attempting fusion. The molecular weight range of PEG may be varied from 1000 to 6000. It gives best results when diluted to from about 20% to about 70% (w/w) in saline or serum-free medium. Exposure to PEG at 37°C for about 30 seconds is preferred in the present case, utilizing murine cells. Extremes of temperature (i.e., about 45°C) are avoided, and preincubation of each component of the fusion system at 37°C prior to fusion gives optimum results.

The successfully fused cells can be separated from the myeloma line by any technique known by the art. The most common and preferred method is to choose a malignant line which is Hypoanthine Guanine Phosphoribosyl Transferase (HGPRT) deficient, which will not grow in an aminopterin-containing medium used to allow only growth of hybrids and which is generally composed of hypoanthine $1x10^{-4}$M, aminopterin $1x10^{-5}$M, and thymidine $3x10^{-5}$M, commonly known as the HAT-containing culture medium immediately after the fusion 24 hours later. The feeding schedules usually entail maintenance in HAT medium for two weeks and then feeding with either regular culture medium or hypoanthine, thymidine-containing medium.

The growing colonies are then tested for the presence of antibodies that recognise the antigenic preparation. Detection of hybridoma antibodies can be performed using an assay where the antigen is bound to a solid support and allowed to react to hybridoma supernatants containing putative antibodies. The presence of antibodies may be detected by "sandwich" techniques using a variety of indicators. Most of the common methods are sufficiently sensitive for use in the range of antibody concentrations secreted during hybrid growth.

Cloning of hybrids can be carried out after 21-23 days of cell growth in selected medium. Cloning can be performed by cell limiting dilution in fluid phase or by directly selecting single cells growing in semi-solid agarose. For limiting dilution, cell suspensions are diluted serially to yield a statistical probability of having only one cell per well. For the agarose technique, hybrids are seeded in a semisolid upper

layer, over a lower layer containing feeder cells. The colonies from the upper layer may be picked up and eventually transferred to wells.

Antibody-secreting hybrids can be grown in various tissue culture flasks, yielding supernatants with variable concentrations of antibodies. In order to obtain higher concentrations, hybrids may be transferred into animals to obtain inflammatory ascites. Antibody-containing ascites can be harvested 8-12 days after intraperitoneal injection. The ascites contain a higher concentration of antibodies but include both monoclonals and immunoglobulins from the inflammatory ascites. Antibody purification may then be achieved by, for example, affinity chromatography.

The presence of oxidatively-modified LDL contemplated herein, in a biological fluid, such as in human plasma can be detected utilizing antibodies prepared as above, either monoclonal or polyclonal, in virtually any type of immunoassay. Alternatively, antibodies specific to oxidized LDL can be detected, using, for example, anti-human antibodies. A wide range of immunoassay techniques are available as can be seen by reference to U.S. Patent Nos. 4,016,043 4,424,279 and 4,018,653. This, of course, includes both single-site and two-site, or "sandwich", assays of the non-competitive types, as well as in the traditional competitive binding assays. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized in a solid substrate and the sample to be tested brought into contact with the bound molecule. For the purpose of the present discussion, oxidatively-modified LDL is referred to as the antigen. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen secondary complex, a second antibody, labelled with a reporter molecule capable of producing a detectable single is then added and incubated, allowing time sufficient for the formation of a tertiary complex of antibody-antigen-labelled antibody (e.g. antibody-oxidatively-modified LDL-antibody). Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may

be quantitated by comparing with a control sample containing known amounts of antigen. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody, or a reverse assay in which the labelled antibody and sample to be tested are first combined, incubated and then added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, and then possible of minor variations will be readily apparent. Alternatively, a two component assay is used comprising the antigen to be detected and a labelled antibody.

In the typical forward sandwich assay, a first antibody having interactivity for oxidatively-modified LDL as contemplated by the present invention, is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs or microplats, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing the molecule to the insoluble carrier. Following binding, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated at 25°C for a period of time sufficient to allow binding of any subunit present in the antibody. The incubation period will vary but will generally be in the range of about 2-40 minutes. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the antigen or first antibody. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody.

By "reporter molecule", as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable or detectable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing

molecules (i.e. radioisotopes). In the case of and enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, streptavidin peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for peroxidase conjugates, 1,2-phenylenediamine, 5-aminosalicyclic acid, or tolidine are commonly used. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody-antigen complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the tertiary complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectophotometricly, to give an indication of the amount of hapten which was present in the sample.

Alternatively, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody absorbs the light energy, inducing a state of excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the ELISA, the fluorescent labelled antibody is allowed to bind to the first antibody-antigen complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength, the fluorescence observed indicates the presence of the antigen of interest, immunofluorescene and ELISA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope,

chemiluminescent or bioluminescent molecules, may also be employed. It will be readily apparent to the skilled technician how to vary the procedure to suit the required purpose. It will also be apparent that the foregoing can be used to detect directly or indirectly (i.e., via antibodies or immune complexes) the LDL of this invention. For example, antibodies to oxidized LDL are readily detectable in biological fluid, e.g. human plasma, by contacting the fluid with oxidized LDL in order to form an immune complex between oxidized LDL and its antibodies. The same techniques are used as described above. Such an immune complex can be detected using labelled anti-mammalian antibodies capable of binding to the antibody in the immune complex.

The present invention is also directed to a kit for the rapid and convenient assay of oxidatively-modified LDL, antibodies thereto or immune complexes thereof in mammalian body fluids, such as human plasma. The kit is compartmentalized to receive a first container adapted to contain an antibody to oxidatively-modified LDL, and a second container adapted to contain a second antibody to said first antibody, said second antibody being labelled with a reporter molecule capable of giving a detectable signal as hereinbefore described. If the reporter molecule is an enzyme, then a third container adapted to contain a substrate for said enzyme is provided. Alternatively, the first container may contain oxidized LDL and the second container contains anti-mammalian (eg. human) antibody labelled with a reporter molecule. In an exemplified use of the subject kit, a sample to be tested for oxidatively-modified LDL, antibodies thereto or an immune complex thereof is contacted to the contents of the first container for a time and under conditions for an LDL antibody complex to form. If such a complex does form, it is detected by the second antibodies of the second container which will bind to the secondary complex to form a tertiary complex and, since said second antibodies are labelled with a reporter molecule, when subjected to a detecting means, the tertiary complex is detected.

For convenience, the "containers" described above may include a microtitre tray or other similar device or may include a paper material.

Accordingly, the present invention provides a rapid and convenient assay for oxidatively-modified LDL, antibodies thereto or immune complexes thereof in biological fluid, such as human plasma. This is particularly important in the assessment of coronary heart disease risk. It is also useful in monitoring therapeutic regima for treating atherosclerosis.

The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a graphical representation of the elution profile of LDL by ion exchange chromatography, ●-● 280nm; o-o 254nm.

Figure 2 is a graphical representation of the fraction spectrum of LDL isolated by ultracentrifugal techniques; Region A: 100µg/ml; Region B: 400µg/ml; Region C: 205µg/ml.

EXAMPLE 1

1.1 Preparation of Native Lipoproteins.

Lipoproteins from normolipidemic individuals are prepared from freshly drawn blood anticoagulated with citrate and ethylenediamine tetracetic acid (EDTA). The plasma density is adjusted to 1.019 with a high density salt solution (NaCl and KBr, containing EDTA) (Hatch et al. Adv. Lipid. Res. 6:1-68, 1968). After centrifugation ($1.7x10^6$gxhours) the pooled superanatant is adjusted to d=1.063, recentrifuged ($2.1x10^6$gxhours) and LDL (d=1.019 to 1.063g/ml) is collected. The preparation is washed and dialysed at 4°C for 24 hours against modified Tyrode's buffer (NaCl 140mM, KCl 2.68mM, $NaHCO_3$ 11.9mM, NaH_2PO_4,H_2O 0.32mM, pH7.4) containing EDTA (0.27m). After sterilization by filtration (0.22mM Millipore membrane), protein concentrations are determined and the preparations concentrated with an Amicon Membrane Concentrator (Modes 202). The protein content is determined by the method of Lowry (Lowry et al. J. Biol. Chem. 193:265-275, 1951).

1.2 Lipoprotein Oxidation.

Lipoprotein are oxidized by incubation LDL (200 microgram protein/mL) with 5 microgram $CuSO_4$ in modified Tyrode's buffer without EDTA for 20 hours at 37°C (Steinbrecher et al. II supra). Oxidation of lipoprotein preparations is assessed as thiobarbituric acid reacting substances (Lowry et al. supra).

1.3 Separation of Oxidatively-Modified Low Density lipoprotein from Human Plasma.

Venous blood is drawn from a normolipidemic female subject receiving 500 microgram bd of the antioxidant drug probucol and native LDL isolated from the plasma by density gradient ultracentrifugation as described above. The LDL is chromatographed on a 2.5 x 22 cm column of DEAE Sepharose CL6b (Pharmacia) equilibrated with 0.04% (w/v) NH_4HCO_3, pH7.4, containing EDTA (0.1 mg/mL) and GSH (0.1 mg/mL). The LDL is eluted with a linear gradient from 0 to 0.8M NaCl prepared with a GM-1 gradient mixer (Pharmacia) at a flow rate of 12 mL/hr. Each 2 mL fraction is monitored at a wavelength of 280 nm for protein and 254 nm for conjugated dienes formed by lipid peroxidation (Pryor et al. Methods Enzymol. 105: 293-299, 1984).

The elution pattern of LDL on the ion exchange column is shown in Figure 1. The elution of LDL, determined by optical density at 254 nm, shows a peak at an NaCl concentration of 0.46M, overlapping the main protein peak, with a larger peak at an NaCl concentration of 0.62M overlapping the smaller protein peak.

1.4 Preparation of an LDL Subfraction.

Blood was collected from patients undergoing coronary angiography with or without angiographically-proven coronary artery disease. Blood was collected into 1mg/ml EDTA, which was subsequently present throughout the isolation of the LDL subfraction.

LDL was isolated from the plasma by isopyonic ultracentriguation followed by density gradient ultracentriguation as described below.

Plasma density was adjusted by 1.090 with solid KBr, overlaid with density solution of 1.090, and spun at 60000rpm, 4.7 hours at 4 °C in a Beckman 60Ti fixed angle rotor. The clearly visable and distinct LDL band was removed and the following gradient produced in Beckman polyallomer ultracentrifuge tubes: pl.102 (4.5mL), LDL at p1.090 (9.0mL), p1.060 (3.0mL), p1.056 (3.0mL), p1.045 (3.0mL, P1.034 (6mL), p1.024 (6mL), p1.019 (3.0mL) and p1.006 (2.25mL). The gradient was spun in a Beckman SW28 rotor at 28000rpm, at 10 °C for 39 hours.

Fractions were collected using upward displacement via a Beckman fraction collection system, and LDL in the density range p1.057-1.063, as determined by an Anton Paar DMA 38 densitometer was pooled and dialysed extensively against PBS and 1mg/mL EDTA at 4 °C. The LDL sub-fraction was then filtered and stored at 4 °C. The elution profile of LDL is shown in Figure 2.

EXAMPLE 2

2.1 Immunization Procedure and Preparation of Monoclonal Antibodies.

Female BALB/c mice 6-8 weeks old are injected with 100 microgram of oxidized LDL in complete Freund's ajuvant (50 microgram subscutaneously and 50 microgram intraperitoneally) 4 weeks apart. Four weeks later, a booster injection of 150 microgram of oxidized LDL in phosphate buffered saline (PBS), pH7.0, is given intraperitoneally. Three days after the booster injection, a single fusion of spleen cells to the mouse myeloma cell line NS1 (Kohler et al. III supra) is performed using polyethylene glycol (Hnatowich et al. J. Nucl. Med. 28:1294-1302, 1987). The resultant hybridomas are screened for antibodies directed against oxidized LDL using an EISA as described in Example 3.1. Positive hybridomas are obtained and cloned. Using this method, hybridoma 4C12 was positive and produced 100 positive colonies on recloning. These clones produce two classes of antibody, IgG2b and IgM as determined by isotyping (CSL Misotype Kit). The hybridomas were further cloned to produce a monoclonal IgG2b designated Mab 216. The clones were then expanded and Mab 216 separated by affinity chromatography on protein A-Sepharose (Pharmacia).

EXAMPLE 3

3.1 Enzyme-linked Immunosorbent Assay.

The hybridomas are screened using an enzyme-linked immunosorbent assay (ELISA). Ninety-six well microtiter plates (Flow Laboratories) are coated with 2mg/ml of oxidatively-modified LDL at 4°C overnight. The plates are then washed four times with PBS containing 0.05% (v/v) Tween 20 and then incubated for 4hr with 200 L of PBS containing 1% (w/v) bovine serum albumin (BSA). The plates are then incubated overnight at 4°C with varying concentrations of the diluted antibody preparation. After washing the plate four times with PBS containing 0.05% (v/v) Tween 20, 100 L of alkaline phosphatase-conjugated affinity-purified goat anti-mouse immunoglobulins (Organon Teknika) at 1:1000 dilution is added and the plates sorted at room temperature for 4 hr. The plates are washed four times with PBS containing 0.05% (v/v) Tween 20 and 100 L p-nitrophenyphosphate, (Sigma Chemicals) (1 mg/mL in 50mM bicarbonate buffer, pH9.8, 1mM Magnesium chloride) (Sigma Chemicals) is added. The plates are stored at room temperature for 1 hr and the reaction terminated by the addition of 3M NaOH. The optical density of the contents of each cell is measured at 405 nm (Titertek Multiskan MCC/340 ELISA reader).

3.2 Biotinylation of the Monoclonal Antibody Mab 216.

A 50% (w/v) ammonium suphate cut of the culture supernatant is dialysed against $NaHCO_3$,pH8.5, for 36 hours and the protein concentration adjusted to 1 mg/mL. The solution is gently stirred on ice and the succinamide ester of biotin (sulfosuccinimidyl 6- (biotinamido) hexanoate, NHS-LC-biotin, Pierce Chemicals) in saline added to a final concentration of 20 microgram of NHS-LC-biotin, Pierce Chemicals) in saline added to a final concentration of 20 microgram of NHS-LC-biotin per mg of protein (Kohler et al. III supra). The reaction is allowed to proceed for 2 hrs and the product is separated from free biotin by dialysis against 2 x 2L of PBS for 24 hrs..

3.3 Sandwich ELISA for Oxidatively Modified LDL.

Microtiter wells are incubated overnight at 4°C with Mab 216 (100 L of culture supernatant), blocked with PBS containing 1% (w/v) BSA for 1 hr at 37°C and washed with PBS containing 0.05% (v/v) Tween 2.0. All subsequent washes are done in the same way. Biotinylated Mab 216 (100 µg/mL) prepared as described in 3.2 is added to each well (100/mL) and incubated for 1 hr at 37°C. The plates are washed and 100 L of streptavidin peroxidase conjugate (BRL) (0.24 µg/mL) is then added to each well and incubated for 60 min. The plates are washed and 100 L of o-phenylenediamine (Sigma Chemicals) dissolved in 25mM sodium citrate/50mM sodium phosphate, pH6, containing 0.4 L/mL H_2O_2 is added. The reaction is terminated after 30 min with 50 L of 4M sulphuric acid and the absorbence determined at 492 nm (See 3.1).

3.4A Detection of Oxidatively Modified LDL in Human Plasma.

The ELISA described in 3.1 above is used to examine fractions obtained from the separation of oxidatively-modified LDL isolated from human plasma (See Example 1.3). The fractions (100 µL) are added to microtiter wells containing adsorbed antibody Mab 216 and the ELISA carried out as described in 3.1. Maximum absorbence at 405 nm corresponds to the well containing the fraction representing oxidatively-modified LDL (Figure 1).

Human LDL treated in vitro with probucol and human LDL oxidized with Cu^{2+} (100 µL) is used to test the cross-reactivity of Mab 216 using the sandwich ELISA described in 3.3. There is minimal colour development with probucol-treated LDL compared to LDL oxidized with Cu^{2+}.

3.4B Detection of Oxidatively Modified LDL in Human Plasma.

The methodology of the present invention includes detection of epitopes lost or gained due to the oxidative event as determined by a panel of monoclonal antibodies to both native and oxidised LDL. Although direct detection of oxidised LDL in plasma would be the most straight forward procedure, other procedures are also

possible and are encompassed by the present invention. Such other procedures include the detection of straight forward total LDL by ELISA and comparing quantitively differences with specific monoclonal antibodies which recognise epitopes sensitive to oxidative processes. This indirect measurement would be tested using procedures as described above.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications which fall within the its spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for detecting oxidatively-modified low density lipoprotein (LDL) in a biological fluid which method comprises contacting the fluid to be tested with an antibody specific to oxidized LDL for a time and under conditions sufficient for an LDL-antibody complex to form and then detecting said complex.

2. The method according to claim 1, wherein the biological fluid is from a mammal.

3. The method according to claim 2, wherein the biological fluid is from a human.

4. The method according to claim 3, wherein the biological fluid is human plasma.

5. The method according to claim 1, wherein the antibody is a monoclonal antibody.

6. The method according to claim 5, wherein the antibody is a murine monoclonal antibody.

7. The method according to claim 6, wherein the monoclonal antibody is specific to oxidized human LDL.

8. The method according to claim 1, wherein the antibody is labelled with a reporter molecule capable of giving a detectable signal.

9. The method according to claim 1, comprising the additional step of contacting the LDL-antibody complex with a second antibody interactive with said first antibody and labelled with a reporter molecule capable of giving a detectable signal for a time

and under conditions sufficient for a LDL-antibody-labelled antibody complex to form.

10. A method for detecting oxidatively-modified low density lipoprotein (LDL) in human plasma which comprises contacting the plasma to be tested with a monoclonal antibody interactive with oxidized-LDL for a time and under conditions sufficient for an LDL-antibody complex to form and then detecting said complex.

11. The method according to claim 10, wherein the antibody is a murine monoclonal antibody.

12. The method according to claim 10, wherein the antibody is labelled with a reporter molecule capable of giving a detectable signal.

13. The method according to claim 10 comprising the additional step of contacting the LDL-antibody complex with a second antibody, said second antibody interactive with said first antibody and labelled with a reporter molecule capable of giving a detectable signal, for a time and under conditions sufficient for an LDL-antibody-labelled antibody complex to form.

14. A method for detecting antibodies interactive with oxidized LDL in a biological fluid which method comprises contacting the fluid to be tested with oxidized LDL for a time and under conditions sufficient to form an antibody LDL complex and the detecting said complex.

15. A method according to claim 14 wherein the biological fluid is human plasma.

16. A method according to claim 14 or 15 wherein the complex is detected by contacting the complex with an antibody labelled with a reporter molecular capable of giving a detectable signal, said antibody capable of binding to oxidized LDL or an antibody in an immune complex with oxidized LDL and then detecting binding of said labelled antibody.

17. A method according to claim 16 wherein the lablelled antibody is a monoclonal antibody.

18. A method for assessing coronary heat disease risk in a human comprising quantitating the level of oxidatively-modified LDL, or oxidized LDL in an immune complex with antibodies thereto in plasma from said human, said method comprising contacting said plasma with a monoclonal antibody specific to oxidized LDL for a time and under conditions sufficient to form an LDL-antibody complex and subjecting said complex to a quantifying detecting means and comparing the level so detected to a predetermined amount.

19. A method for assessing coronary heat disease risk in a human comprising quantitating the level of antibodies specific for oxidatively-modified LDL or antibodies in an immune complex with oxidized LDL in plasma from said human, said method comprising contacting said plasma with oxidized LDL for a time and under conditions sufficient to form an antibody-LDL complex and subjecting said complex to a quantifying detecting means and comparing the level so determined to a predetermined amount.

20. A kit for the detection of oxidatively-modified LDL, said kit comprising in compartmentalized form a first contain adapted to contain an antibody interactive with oxidized LDL and a second contain adapted to contain a second antibody interactive wiht said first antibody and labelled with a reporter molecular capable of giving an identifiable signal.

21. A kit according to claim 20 wherein said second antibody is labelled with an enzyme and wherein said kit further comprises a third container adapted to contain a substrate for said enzyme.

22. A kit for the detection of antibodies ineractive with oxidatively-modified LDL, said kit comprising in compartmental form a fist container adapted to contain oxidized LDL and a second container adapted to contain an antibody labelled with

a reporter molecular capable of giving an identifiable signal and interactive with said antibody interactive with oxidized LDL.

23. A kit according to claim 22 wherein said labelled antibody is an anti-human antibody.

24. A kit according to claim 22 or 23 wherein the reporter molecular is an enzyme and said kit further comprises a third container adapted to contain a substrate for said enzyme.

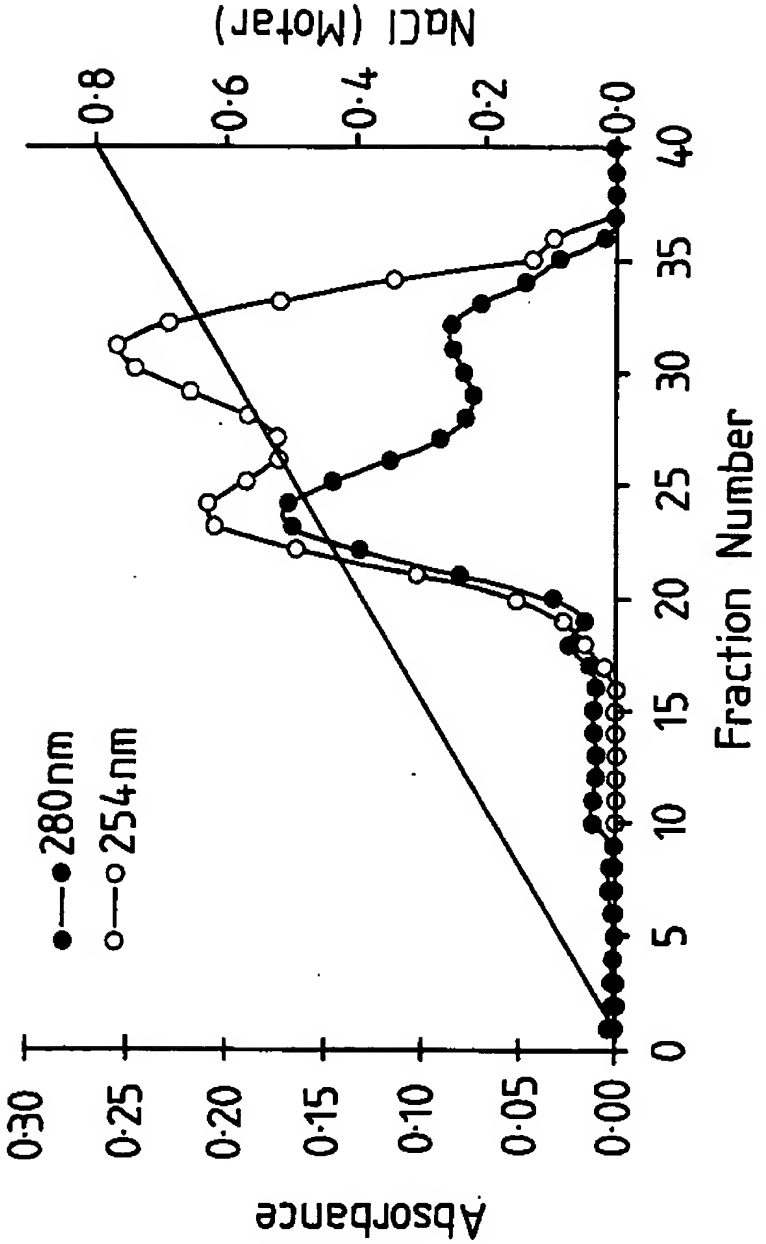
Absorbance
0·30
0·25
0·20
0·15
0·10
0·05
0·00
0
5
10
15
20
25
30
35
40
Fraction Number
NaCl (Motar)
0·8
0·6
0·4
0·2
0·0
280nm
254nm

FIG 1

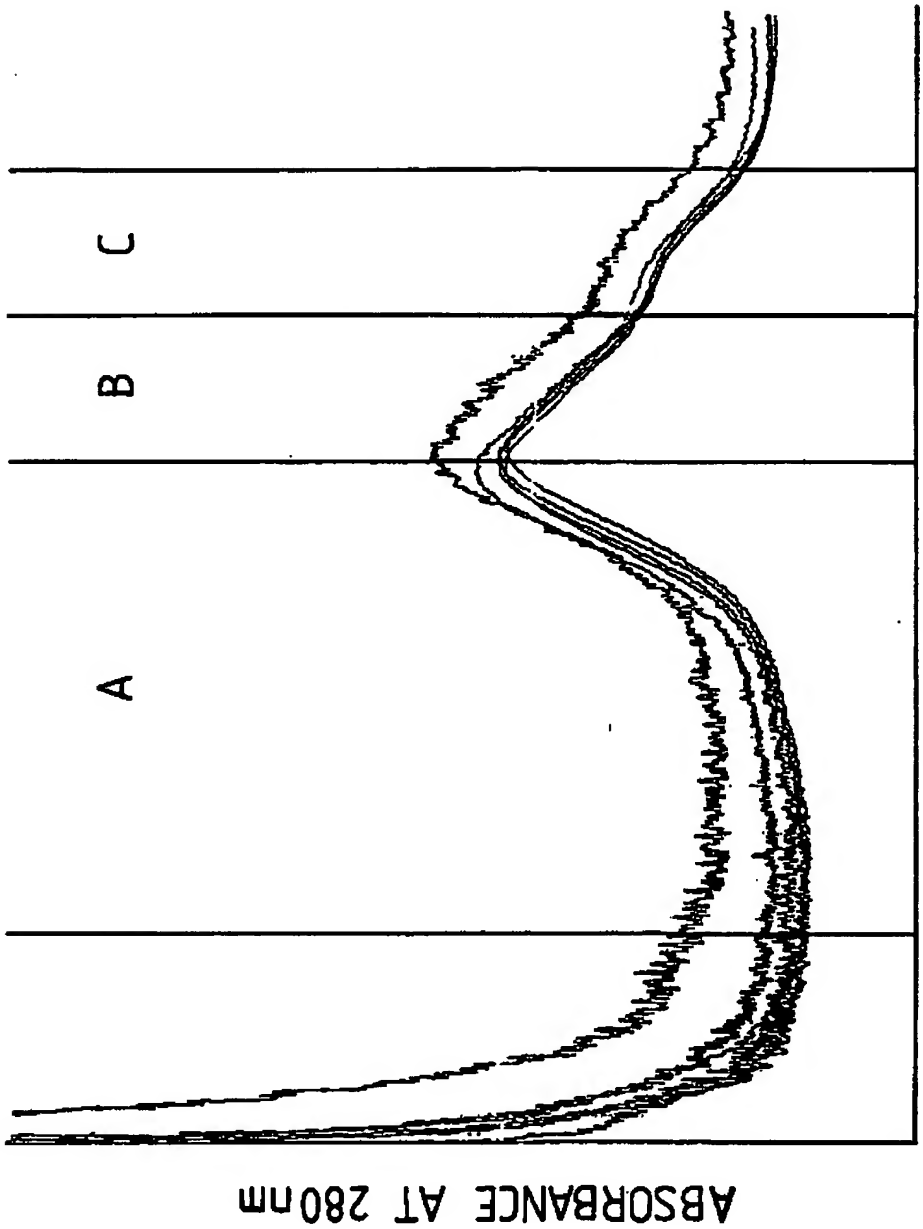

FIG 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 94/00171

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[5] G01N 033/68, 033/577, C12P 021/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT AND CHEM ABS
SEE DETAILS IN ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU C12P 021/08

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT WPAT DATABASE, KEYWORDS:OXID:(S)LOW(W)DENSITY(W)LIPOPROTEIN # OR LDL(S)OXID:
CHEMICAL ABSTRACTS DATABASE, KEYWORDS:(OXIDI:OR OXIDA:)(S)(LOW()DENSITY()LIPOPROTEIN # OR LDL) AND 15/CC AND 89-94 AND NOT P/DT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
PX	CLINICA CHIMICA Acta (1993) volume 218 pages 97-103 (Hualiang Wang et al) "Quantitation of plasma oxidatively modified low density lipoprotein by sandwich enzyme linked immuno sorbent assay". See page 98	1-22
X	ARTERIOSCLEROSIS (1990), volume 10 no 3, pages 325-334 (Wulf Palinski et al) "Antisera and Monoclonal antibodies specific for Epitopes generated during Oxidative Modification of Low Density Lipoprotein". See page 331 and 334	1-22

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
	4 August 1994 (04.08.94)

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	AU-A-85987/91 (DAIICHI PURE CHEMICALS CO LTD) Published 14 May 1992 (14.05.92). See whole article	1-22
Y	AU 70452/91 (THE BETH ISRAEL HOSPITAL ASSOCIATION) Published 2 May 1991 (02.05.91). See whole article	1-22
Y	Derwent Abstract Accession No. 87-361926/51, Class S03, SU,A,1312-486 (A MED CARDIOLOGY) 23 May 1987 (23.05.87)	1-22

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	91/85987	EP	484863	JP	4173096		
AU	91/70452	BR	9007721	BR	9007725	CA	2067356
		CA	2067364	EP	494992	EP	495014
		FI	921430	FI	921431	WO	9104744
		WO	9105536				

END OF ANNEX